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**(54) Title:** METHODS AND COMPOSITIONS FOR SUPPRESSING ALLOGRAFT REJECTION IN MAMMALS

**(57) Abstract**

Disclosed herein are methods for suppressing allograft rejection in mammals comprising administering to a mammal about to undergo or having undergone allograft surgery an agent selected from the group consisting of splenic tissue from an allograft donor, splenic extracts, cultured lymphocytes from an allograft donor, extracts of said cultured lymphocytes, MHC antigens, transplantation rejection suppressive fragments and analogs of MHC antigens in an oral or aerosol form. Also disclosed herein are pharmaceutical formulations and dosage forms for use in said methods.

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**METHODS AND COMPOSITIONS FOR SUPPRESSING  
ALLOGRAFT REJECTION IN MAMMALS**

15

**FIELD OF THE INVENTION**

This invention relates to methods and compositions for suppressing the immune response in animals. More particularly, but not by way of limitation, the present invention is directed to pharmaceutical formulations and methods for suppressing and controlling the immune response of mammals against the introduction of foreign tissue. The invention also includes methods for prolonging the survival of transplanted organs and tissues.

**BACKGROUND OF THE INVENTION**

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The success of surgical transplantation of organs and tissue is largely dependent on the ability of the clinician to modulate the immune response of the transplant recipient. Specifically the immunological response directed against the transplanted foreign tissue must be controlled if the tissue is to survive and function. Currently, skin, kidney, liver, pancreas and heart are the major organs or tissues with which allogeneic transplantations are performed. It has long been known that the normally functioning immune system of the transplant recipient recognizes the transplanted organ as "non-

self" tissue and thereafter mounts an immune response to the presence of the transplanted organ. Left unchecked, the immune response will generate a plurality of cells and proteins that will ultimately result in the loss of biological functioning or 5 the death of the transplanted organ.

Tissue and organ transplant recipients are customarily treated with one or more cytotoxic agents in an effort to suppress the transplant recipient's immune response against the transplanted organ or tissue. For example, cyclosporine 10 (cyclosporin A), a cyclic polypeptide consisting of 11 amino acid residues and produced by the fungus species *Tolypocladium inflatum* Gams, is currently the drug of choice for administration to the recipients of allogeneic kidney, liver, pancreas and heart (i.e., wherein donor and recipient are of the same 15 species of mammals) transplants. However, administration of cyclosporine is not without drawbacks as the drug can cause kidney and liver toxicity as well as hypertension. Moreover, use of cyclosporine can lead to malignancies (such as lymphoma) and lead to opportunistic infection due to the "global" nature 20 of the immunosuppression it induces in patients receiving long term treatment with the drug, i.e., the host's normal protective immune response to pathogenic microorganisms is downregulated thereby increasing the risk of infections caused by these agents.

25 Preliminary results have shown FK-506 (which has a similar mode of action as cyclosporine) to be as potent as cyclosporine in its immunosuppressive qualities and to have fewer toxic side effects than cyclosporine. However, because

studies on FK-506 are only in the early stages, it is not available to the general population. Hence, the use of this agent is limited.

Other drugs and/or therapies which are currently administered (either in conjunction with cyclosporine or alone) to suppress the rejection of allogeneic grafts or allografts are also non-specific immunosuppressive drugs or therapies. Steroids, such as prednisone and methylprednisolone, and Azathioprine (an analog of 6-mercaptopurine) are among the non-specific immunosuppressive drugs used to prolong allograft survival in transplantation recipients.

OKT3 monoclonal antibodies, directed against the CD3 antigen present on T-cells, have also been employed as non-specific immunosuppressive therapeutic agents in allograft recipients. However, OKT3 monoclonal antibodies are of murine origin and the patients to whom such monoclonal antibodies are given mount an immune response against these foreign proteins. Thus the usefulness of such materials is limited.

Another drawback to the above-mentioned drugs and antibodies is that they must be administered indefinitely to suppress allogeneic graft rejection, and tolerance to the foreign tissue does not develop.

Total lymphoid irradiation (TLI) is yet another form of non-specific immunosuppressive therapy that has been used clinically and experimentally to prolong allograft survival. The radiation exposure and treatment schedule for TLI were developed for the treatment of Hodgkin's disease and were subsequently found to be immunosuppressive. Although, TLI

induces production of the "global" immunosuppression mentioned above and has the same limitations of other global immunosuppressive therapies, it is the only form of immunosuppression currently in use which appears to induce a specific tolerance 5 to allogeneic tissue. However, TLI is cumbersome to administer and is in an early stage of development, and thus its usefulness is limited.

The oral and aerosol administration of antigens has also been recognized as an effective way to suppress the immune 10 response in mammals to these antigens. The advantages of administering antigens via the oral route include: the simplicity of the techniques involved; the convenience of such techniques since many of the methods can be developed in-situ at the research or treatment facility; the safe, non-toxic 15 effects of the ingestion route; and the specificity that can be provided with the antigens.

Recent studies on several autoimmune disease models have demonstrated that the oral administration of antigens can suppress at least the portion of the immune response that is 20 directed against autoantigens and also protect the treated animals from the induction of specific autoimmune diseases. For example, various animal models are available for the study of Type 1 diabetes as an autoimmune disorder. These include the BB rat (Nakbookda, A.F., et al., Diabetologic 14: 199-207, 25 1978) and the NOD (non-obese diabetic) mouse in which diabetes develops spontaneously (Prochazka et al. Science 237:286, 1987). Islet-cell specific, CD4- and CD8-positive T-lymphocytes have been implicated as the causative agents respon-

sible for damage to islet beta cells, as demonstrated by transfer of lymphocytes from affected adults to newborn animals (J. Exp. Med. 166:823, 1987).

5       Experimental allergic encephalomyelitis (EAE) is an induced T-cell mediated autoimmune disease directed against myelin basic protein (MBP) that is widely used as an animal model for the human disease Multiple Sclerosis (MS). EAE can be induced in small mammals by intravenous administration of MBP and a strong adjuvant, such as Freund's complete adjuvant. 10      This treatment induces an acute, monophasic autoimmune disease with the characteristics of MS.

Weiner et al., U.S. Patent Application entitled Method Of Treating Or Preventing Type 1 Diabetes By Oral Administration Of Insulin, filed October 10, 1990, discloses oral and aerosol compositions and pharmaceutical formulations containing insulin which are useful for treating mammals suffering from or at risk for autoimmune diseases having the characteristics of Type 1 diabetes.

20      Weiner et al., U.S. Patent Application Ser. No. 460,852 filed February 21, 1990, (the national stage of PCT Application No. PCT/US88/02139, filed June 24, 1988), which is a continuation-in-part application of U.S. Patent Application Ser. No. 065,734 filed June 24, 1987, generally discloses the treatment of autoimmune diseases by oral administration of autoantigens.

25      Weiner et al., U.S. Patent Application Ser. No. 454,486 filed December 20, 1989, discloses the aerosol administration of autoantigens, disease-suppressive fragments of said

autoantigens and analogs thereof as an effective method for treating T-cell mediated autoimmune diseases.

Weiner et al., U.S. Patent Application Ser. No. 487,732, filed March 2, 1990, discloses synergists (enhancers) 5 for use with oral administration of autoantigens, disease suppressive fragments and analogs thereof as effective treatments for T-cell mediated autoimmune diseases.

Weiner et al., U.S. Patent Application Ser. No. 551,632 filed July 10, 1990, a continuation-in-part of U.S. Patent 10 Application Serial No. 379,778, filed July 14, 1989, discloses methods of preventing or treating uveoretinitis in mammals by oral administration of purified S antigen, Interphotoreceptor Retinoid Binding Protein (IRBP) antigen or disease suppressive fragments thereof.

15 Nagler-Anderson, et al., (Proc. Natl. Acad. Sci. (USA) 83: 7443-7446, 1986), describe the oral administration of collagen to suppress collagen-induced arthritis in a mouse model.

20 However, the above-mentioned references do not disclose the use of antigens to suppress the mammalian graft rejection mechanism because it has not been shown that the principle of oral administration of transplantation antigens could prevent allograft rejection.

25 The present invention proposes the clinical administration to mammalian graft recipients of alloantigens via oral and aerosol routes to induce a tolerance to foreign tissue grafts. The invention will be primarily useful in the field of organ transplantation including bone marrow. Although previous

studies have shown that alloantigens injected intravenously to recipients can prolong the survival of renal transplants (Transplantation 39:56, 1985; J. Immunol. 121:1480, 1978; J. Exp. Med. 149:1042, 1979), no disclosure or suggestion of introducing these antigens orally or in an aerosol form was made therein.

It is, therefore, an object of the present invention to provide agents and methods for suppressing the detrimental immune response in mammals to the grafting or transplantation of foreign (or "non-self") tissues and organs.

Another object of the present invention is to provide pharmaceutical formulations and preparations that may be administered to mammals to suppress the immune rejection of surgically transplanted tissues.

A still further object of the invention is to provide synthetic compositions and pharmaceutical formulations that may be administered to mammals via the oral or aerosol route to suppress the mammalian immune response to the presence of transplanted tissue or organs.

These and other objects of the present invention will become apparent to those of ordinary skill in the art in light of the following.

#### SUMMARY OF THE INVENTION

It has now been unexpectedly discovered that compositions comprising specific antigenic agents, including by way of non-limiting example allogeneic spleen tissue and cultured lymphocytes and specific Major Histocompatibility Complex (MHC) antigens can be administered to mammals via the oral or aerosol

route to suppress the mammalian immune response to surgically transplanted "non-self" organs or tissues. Because the effect is dependent upon MHC molecules present on the surface of spleen cells, which differ between the tissue donor and the 5 recipient, administration of these antigens alone is expected to be effective.

Orally administered allogeneic splenocytes can suppress the immune response of a host mammal which normally occurs shortly after transplant surgery against surgically transplanted 10 "non-self" tissue in an antigen-specific manner. It has also been found that oral ingestion of allogeneic spleen tissue preparations depresses the delayed type hypersensitivity reaction and mixed lymphocyte reaction in mammals. Compositions and pharmaceutical formulations for oral administration 15 of allogeneic splenocytes may be prepared from natural allogeneic tissue. For administration to humans such compositions comprise synthetic derivatives of antigens i.e., peptide fragments of MHC antigens.

In practicing the method of the present invention, 20 pharmaceutical formulations containing synthetic antigens or natural allogeneic splenic or lymphocyte tissue or cell derivatives are prepared and orally administered to mammalian subjects some time prior to organ or tissue transplant surgery.

Additionally, an aerosol delivery system can be 25 prepared with essentially the dosages of splenocyte derivatives or MHC antigens as above and a pharmaceutically suitable carrier or diluent. The aer sol formulations can also be administered sometime prior to transplant surgery via the

aerosol route. These and other improvements will be described in the following descriptions, drawings and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 is a graph showing the effect of feeding allogeneic splenocytes on the mixed lymphocyte reaction (MLR).

Figure 2 is a graph showing the effect of feeding allogeneic splenocyte lysates on the mixed lymphocyte reaction.

Figure 3 is a graph showing the kinetics of oral tolerance to alloantigens.

10 Figure 4 is a graph depicting the effect of feeding syngeneic or allogeneic splenocytes on delayed type hypersensitivity (DTH) reactions.

15 Figure 5 are a series of immunofluorescence analyses (histograms) showing the effect of feeding allogeneic splenocytes on lymphocyte composition.

Figure 6 is a graph showing the survival of cardiac allografts in control rats, LEW rats fed syngeneic splenocytes, LEW rats fed third party (WF) splenocytes or LEW rats fed allogeneic splenocytes.

20 Figure 7 is a graph showing the effects of feeding splenocytes on the MLR of skin graft recipients compared to control (non-fed) skin graft recipients.

DETAILED DESCRIPTION OF THE INVENTION

25 The contents of all patent applications, patents and literature references referred to in this specification are hereby incorporated by reference in their entirety.

The present invention addresses the need for an alternate to existing methods for suppressing the immune response directed against foreign tissue transplants, as for example, post-transplant surgery. In addition, the methods of 5 the present invention provide for prolonged survival of organ and tissue allogeneic grafts (i.e. transplants from individuals of the same species) in a mammal in need of such treatment.

Thus, the present invention provides means whereby the 10 rejection of tissue allografts can be prevented, thus prolonging the survival of transplanted tissue and organs.

It has now been unexpectedly discovered that oral administration of allogeneic splenocytes or synthetic MHC 15 antigens (or immune suppressive fragments or analogs thereof) is effective for suppressing the in vitro mixed lymphocyte reaction which is a model system for the graft rejection response in post-transplant mammalian recipients.

Without wishing to be bound to any particular theory of operation or mechanism of action for the invention it is believed that the oral administration of allogeneic splenocytes 20 or derivatives of MHC antigens pursuant to the present invention affects the immunological mechanisms of graft rejection, i.e. the activation of helper T-cells is decreased by the induction of specific suppressor T-cells.

In the following discussions the following terms shall 25 have the meaning ascribed to them below.

"Oral administration" shall mean both oral administration and enteral administration (delivery directly into the stomach).

"Mammal" shall mean any organism having an immune system and therefore susceptible to allogeneic graft rejection.

5 "Aerosol" refers to finely divided solid or liquid particles that may be created using a pressurized system such as a nebulizer. The liquid or solid source material contains MHC antigens and/or disease suppressive fragments and analogs thereof as defined herein.

The "aerosol route" of administration means delivery of an aerosol formulation to a host via the nasal or oral airway.

10 "Major Histocompatibility Complex" (MHC) is defined as a complex series of mammalian cell surface proteins. The MHC plays a central role in many aspects of immunity both in presenting histocompatibility (or transplantation) antigens and in regulating the immune response against conventional 15 (foreign) antigens. There are two types of MHC protein molecules, Class I and Class II. Class I MHC proteins are present on virtually all tissues and Class II MHC proteins are present on the surface of activated T-cells, macrophages and other immune system cells. The human MHC genes (the HLA 20 genetic locus) are located on human chromosome 6, the mouse MHC genes are located in the H-2 genetic locus on mouse chromosome 17 the analogous rat MHC genes are referred to as RTI.

25 "Class I MHC antigens" are defined as membrane glycoproteins present on the surface of all nucleated cells and play a key role in antigen recognition by CD8+ cytotoxic T-cells.

"Class II MHC molecules" are membrane glycoproteins that form part of the MHC and are most important in the

initiation of immune responses. Class II MHC molecules are found mainly on cells of the immune system including B-cells, macrophages, brain astrocytes, epidermal Langerhan's cells, dendritic cells, thymic epithelium and helper T-cells. Class 5 II MHC molecules are involved in regulating the immune response during tissue graft rejection, stimulation of antibody production, graft-versus-host reactions and in the recognition of "self" (or autologous) antigens, among other phenomena.

"MHC antigens" are defined herein as Class I and/or 10 Class II MHC antigens. MHC antigens of the present invention include both Class I and Class II, either alone or in combination.

"Allogeneic tissue extracts" are defined as splenocyte, 15 splenic tissue or cultured lymphocyte extracts obtained from an allogeneic transplant donor and prepared as described below.

"Immune suppressive fragments" means any peptide or polypeptide containing partial amino acid sequences or moieties of analogs of the relevant MHC antigens possessing the ability to induce suppression of the hosts immune response against 20 organ or tissue allogeneic grafts. Such fragments need not possess the alloantigenic properties of the entire MHC molecule.

"Analogs" of immune suppressive fragments refers to 25 compounds that are structurally related to suppressive fragments of MHC antigens thereof which possess the same biologic activity, i.e., the ability to suppresses a mammalian hosts response against a transplanted organ or tissue. The term includes peptides having amino acid sequences which differ from

the amino acid sequence of the relevant MHC antigens of the potential graft recipient by one or more amino acid residues.

Disease suppressive fragments and analogs for use in the present invention can be synthesized using well known solid 5 phase synthesis techniques (Merrifield, R.B. Fed. Proc. Am. Soc. Ex. Biol. 21: 412, 1962 and J. Am. Chem. Soc. 85: 2149, 1963; Mitchel, A.R. et al., J. Am. Chem. Soc. 98: 7357, 1976; Tam, J. et al., J. Am. Chem. Soc. 105: 6442, 1983). Analogs can be constructed by identifying an equivalent amino acid 10 sequence and using the peptide synthesis techniques disclosed above.

Analogs can be provided using the known amino acid sequence of MHC antigens as disclosed in Immunogenetics 29:231-234, 1989.

15 Disease-suppressive analogs and fragments can also be obtained using recombinant DNA techniques that are well-known in the art.

Disease suppressive fragments of MHC antigens and 20 analogs thereof can be identified using routine experimentation using suitable in vivo systems such as those of Examples 1-4 below.

T-lymphocytes can be obtained from a potential allograft donor using methods well known in the art and cultured as described in Transplantation 41:549, 1986 and Transplantation 48:639, 1989 and administered to a mammal about to undergo 25 or having undergone (as described below) an organ or tissue allograft.

Extracts (or lysates) of splenic tissue or cultured lymphocytes can be prepared using techniques well known in the art such as those described in Example 1 below.

In accordance with the present invention, conventional tissue typing, well-known in the art and routinely conducted on all transplant donors and recipients, is performed on a potential transplant donor to determine the MHC phenotype of the donor tissue or organ. Synthetic MHC antigens, disease suppressive fragments or their analogs can then be synthesized using the techniques described above. These antigens and/or fragments may be administered to mammals, especially humans, who are to receive a transplant; or to patients that have already received transplanted "non-self" tissue. The methods and compositions of the present invention may be used to treat mammals that have previously received "non-self" organ or tissue transplants and are beginning to display the initial symptoms of allograft rejection (such as fever, tenderness of the transplanted organ or loss of function thereof). The method and compositions of the invention are useful to preserve the organ or tissue and damp down or shut off that portion of the immune response of the recipient that is directed against the transplanted tissue or organ. To be effective the compositions and methods of the present invention must be administered before total rejection occurs.

Pursuant to the present invention, MHC antigens or transplantation rejection suppressive fragments or their analogs are ingested by a mammal that is to receive, or has already received a "non-self" organ or tissue transplant via

the oral or enteral route, in an amount of between about 0.1 mg per kg body weight and about 10 mg per kg of body weight per day. The pharmaceutical compositions of the invention may be administered as a single dose or in multiple dose form via the 5 oral or enteral route. Preferably, the is administered in an amount between about 1 mg and about 5 mg per kg body weight of said mammal per day. The exact amount to be administered will vary depending on the severity and stage of a patient's disease and the physical condition of the patient.

10 When administering splenic cells, cultured lymphocytes or extracts thereof, between about  $10^6$  and about  $10^9$  cell equivalents per kg body weight per day may be administered in single or divided doses.

15 The timing of such treatments shall be such that, if possible, the pharmaceutical formulations or dosage forms of the present invention are administered between about 7 and about 14 days before the transplantation is performed. The treatment is preferably continued for at least about 6 months after the transplanted organ or tissue has been introduced into 20 the host (recipient) organism and may be continued indefinitely if necessary or desirable.

25 In addition, if a transplant recipient (either already receiving the compositions of the invention or not) begins to manifest symptoms of rejection, the pharmaceutical formulations of the present invention may be administered in increased amounts and/or frequency.

The present invention also is directed to oral dosage forms and pharmaceutical formulations for administration to mammals in order to prolong the survival of or suppress the rejection of a transplanted organ or tissue. It will be 5 understood that any statistically significant prolongation in graft survival pursuant to the treatment of the present invention is within the scope of the invention.

The oral pharmaceutical formulations of the present invention may also contain inert constituents including 10 pharmaceutically acceptable carriers, diluents, fillers, solubilizing or emulsifying agents and salts of the type that are well-known in the art. For example, tablets and caplets may be formulated in accordance with conventional procedures employing solid carriers, such as starch and bentonite, that 15 are well-known in the art. Examples of solid carriers include bentonite, silica, dextrose and other commonly used carriers. Further non-limiting examples of carriers and diluents which may be used in the formulations of the present invention include saline and any physiologically buffered saline solution 20 such as phosphate buffered saline, pH 7-8 and water.

Capsules employed in the present invention may be made from any pharmaceutically acceptable material such as gelatin or cellulose derivatives. The active biological materials of the invention may be administered in the form of sustained 25 release oral delivery systems and/or enteric coated oral dosage forms such as those described in U.S. Patent No. 4,704,292 issued November 3, 1987, U.S. Patent No. 4,309,404 issued

January 5, 1982 and U.S. Pat. No. 4,309,406 issued January 5, 1982.

It will be appreciated that the unit content of active ingredient or ingredients contained in an individual dose of 5 each dosage form need not in itself constitute an effective amount for suppressing graft rejection since the necessary effective amount can be reached by administration of a plurality of dosage units.

The preferred route of administration of the dosage 10 forms of the present invention is orally or enterally. Preferred oral or enteral pharmaceutical formulations or dosage forms may comprise for example, between about 70 mg and about 500 mg of MHC antigens, disease suppressive fragments or analogs thereof or between about  $10^7$ - $10^{10}$  cell equivalents when 15 using allogenic cells or extracts thereof.

In an alternative embodiment of the present invention the pharmaceutical formulations of the present invention are administered to mammals in aerosol form. It is anticipated that smaller quantities of the allogeneic tissue extracts or 20 MHC antigens, disease suppressive fragments or their analogs will be required to achieve suppression of graft rejection when using the aerosol form of administration. This has been found to be the case in treating experimental allergic encephalomyelitis (EAE) with myelin basic protein (MBP), and also 25 in treating adjuvant arthritis with collagen as disclosed in the co-pending U.S. Patent Application of Weiner et al. Ser. No. 454,486 filed December 20, 1989. The quantity of MHC antigens, disease suppressive fragments or the analogs of such

materials which may be administered in an aerosol dosage form would be between about 0.01 mg and 10 mg per kg body weight of a mammal per day. The aerosol dosage forms of the present invention may be administered to a patient via the aerosol route 5 in a single dosage form or multiple dosage forms. The exact amount to be administered will vary depending on the state and severity of a patient's disease, the activity of the patient's immune system and the physical condition of the patient.

When administering splenic cells, cultured lymphocytes 10 or extracts thereof, between about  $10^5$  and about  $10^9$  cell equivalents per kg body weight per day may be administered in single or divided doses in an aerosol form.

The aerosol pharmaceutical formulations of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are well-known in the art. Specific non-limiting examples of the carriers and/or diluents 15 that are useful in the aerosol pharmaceutical formulations of the present invention include water, normal saline and physiologically-acceptable buffered saline solutions such as 20 phosphate buffered saline solutions, pH 7.0-8.0.

Examples of useful solubilizing and emulsifying agents are physiologically balanced salt solutions, phosphate buffered saline and isotonic saline. The salts that may be employed in 25 preparing the aerosol dosage forms of the invention include the pharmaceutically acceptable salts of sodium and potassium.

The route of administration of allogeneic spleen cells, cultured lymphocytes extracts thereof or MHC antigen or disease

5 suppressive fragments or their analogs according to this alternate embodiment of the present invention is in an aerosol or inhaled form. The aerosol compositions of the present invention can be administered as a dry powder or in an aqueous solution. Preferred aerosol pharmaceutical formulations may comprise, for example, a physiologically-acceptable buffered saline solution containing between about 7 mg and about 700 mg of the compositions of the present invention, disease suppressive fragments or analogs thereof.

10 Dry aerosol in the form of finely divided solid particles of tissue extracts from spleen cells, MHC antigens disease suppressive fragments or analogs thereof that are not dissolved or suspended in a liquid are also useful in the practice of the present invention. The compositions of the 15 present invention may be in the form of dusting powders and comprise finely divided particles having an average particle size of between about 1 and 5 microns, preferably between 2 and 3 microns. Finely divided particles may be prepared by pulverization and screen filtration using conventional techniques that are well known to those skilled in the art. The particles 20 may be administered by inhaling a predetermined quantity of the finely divided material, which can be in the form of a dry atomized powder.

25 The pharmaceutical formulations of the present invention may be administered via the aerosol route by means of a nebulizer, as an example those described in U.S. Patent Nos. 4,624,251 issued November 25, 1986; 3,703,173 issued November 21, 1972; 3,561,444 issued February 9, 1971 and 4,635,627

issued January 13, 1971. The aerosol material is inhaled by the subject to be treated.

Other systems of aerosol delivery, including for example the pressurized metered dose inhaler (MDI) and the dry 5 powder inhaler as disclosed in Newman, S.P. in Aerosols and the Lung, Clarke, S.W. and Davia, D. eds. pp. 197-224, Butterworths, London, England, 1984 can be used in conjunction with the method of the present invention.

Aerosol delivery systems of the type disclosed herein 10 are available from numerous commercial sources including Fisons Corporation (Bedford, MA), Schering Corp. (Kenilworth, NJ) and American Pharmoseal Co., (Valencia, CA).

In accordance with the present invention, experiments 15 were performed in which the effects of oral administration of allogeneic splenocytes to Lewis rats were studied, with particular attention being given to the effects on the immune response of the transplant recipient. To this end, the in vitro mixed lymphocyte response (MLR), the delayed type hypersensitivity (DTH) reaction, and the in vivo accelerated 20 cardiac allograft rejection techniques were utilized. In each case, the oral administration (to the recipient of a "non-self" tissue transplant) of splenocyte cells from a donor animal resulted in suppression of these T-cell mediated immune reactions. As T-cells have been implicated as the major 25 mediators of allograft rejection, the results of these tests establish the practical efficacy of the methods and pharmaceutical formulations of the present invention.

The present invention is illustrated in specific working examples presented below which are intended to illustrate the present invention without limiting the scope thereof.

5

EXAMPLE 1: PREPARATION OF MATERIALS AND TEST SUBJECTS

1. Subject Mammals

The test population was comprised of male rats of the Lewis (LEW), Wistar Furth (WF) and Brown Norway (BN) variety 10 (obtained from Harlan Sprague Dawley Inc., Indianapolis, IN). The rats in the experiments described below were approximately 8-10 weeks old, and were bred under careful observation.

15

2. Preparation of splenocytes for oral administration

Fresh splenic tissue was obtained from syngeneic (same species, same strain) or allogeneic (same species, different strain) animals shortly prior to oral administration. Single cell splenocyte suspensions were prepared by mashing the fresh 20 spleen through a standard stainless steel mesh (2 inches by 2 inches). Red blood cells were specifically lysed with Tris-ammonium chloride buffer according to standard procedures well known in the art, washed twice with Hank's Balanced Salt Solution (HBSS) and resuspended into various concentrations as 25 described below before use.

3. Preparation of splenocyte lysate

Splenocytes prepared as in the above method were lysed by repetitive freeze-thawing in the following manner:

(a) Cells were quick frozen at -70°C for 30 minutes;

(b) Quick frozen splenocytes were then thawed at 37°C;

5 (c) This freeze-thaw cycle was repeated one more time.

The resulting materials were used for oral administration.

10 EXAMPLE 2: ORAL ADMINISTRATION OF PREPARED SPLENOCYTE SUSPENSION

A one milliliter dose of the cell suspension as prepared in Example 1, was orally introduced to each test rat with a syringe having a ball-tipped feeding needle (Thomas Scientific, Swedesboro, NJ).

15 The following laboratory immunological and pathological procedures were conducted on the lymphatic organs of the test rats.

EXAMPLE 3: MIXED LYMPHOCYTE REACTION

20 Cervical lymph nodes were taken from the responder (LEW) and the stimulator (WF or BN) rats. The excised nodes were then pressed through stainless steel mesh as above and suspended in Phosphate Buffered Saline.

25 The isolated lymph node cells were then washed twice and resuspended into RPMI 1640 medium, containing 10% fetal calf serum (FCS), 1% penicillin and streptomycin (Microbiological Associates, Walkersville, MA)  $2 \times 10^{-5}$  M 2-mercaptoethanol, and 5 mM HEPES, at a concentration of  $6 \times 10^6$  cells/ml.

Responder cells were seeded into a 96-well flat-bottomed

culture plates (Costar Cambridge, MA) at 50 microliters per well, with or without irradiated stimulator cells (3000 Rads gamma irradiated using a Shepherd irradiator, Model 143-45 and a Cesium-137 source) of the same volume.

5 The treated cells were then cultured at 37°C with 5% CO<sub>2</sub> for four days before they were pulsed for 6 hours with <sup>3</sup>H-thymidine (1 microCi/well, obtained from NEN Dupont, Boston, MA). Cell proliferation was monitored by incorporation of <sup>3</sup>H-thymidine measured by a Beckman liquid scintillation counter.

10 SUPPRESSOR ASSAY

Obtained lymph node cells were irradiated (1000 Rads of gamma radiation) and added to a test MLR at concentrations varying from 5 to 20% of total cells per well (experimental wells). Control wells were set up with no modulators while 15 background wells had only responder cells. These cultures were incubated at 37°C and in 5% CO<sub>2</sub> for 96 hours. Proliferation was assayed by pulsing the plates with 1 microCi/well <sup>3</sup>H-thymidine for the last 6 hours of culture. The plates were then harvested as described above.

20 DELAYED TYPE HYPERSENSITIVITY DTH REACTIONS

Rats of each group were immunized subcutaneously in the footpad with 10 million gamma irradiated (3000 RAD) allogeneic splenocytes. Ten days later, they were injected again with the same dosage in the ear lobe. The responses were determined as 25 the changes in the ear thickness before and 48 hours after the challenge.

CELL TYPING

The phenotypes of the extracted lymphocytes were tested by indirect immunofluorescent staining and with a fluorescence-activated cell sorter (FACS). The lymph cells were first 5 incubated for 1 hour with primary monoclonal antibodies against the cell surface markers CD4 or CD8, or mouse immunoglobulin (Organon-Teknica, Westchester, PA) and washed twice with PBS containing 0.02% sodium azide. They were then further incubated with FITC-conjugated goat-anti-mouse IgG (1:40) 10 (Organon Teknica) in the dark for 30 minutes and in the presence of 15% autologous normal rat serum. The cells were thoroughly washed and fixed with 1% formaldehyde before testing.

15 Additionally, surgical transplant methods of the type described in the following example were performed.

EXAMPLE 4: CARDIAC ALLOGRAFT

LEW rats were subjected to surgical transplant procedures. An accelerated rejection model was used wherein 20 LEW strain rats were pre-sensitized with BN strain full-thickness skin grafts seven days before the cardiac allograft, with and without oral ingestion of splenocyte preparations.

25 Seven days later, a (LEW $\times$ BN)F1 strain test vascularized cardiac allograft was performed on each pre-treated rat. The cardiac grafts were anastomosed to the infra-renal abdominal aorta. Rejection was defined as complete cessation of heart beat as determined by daily palpation of the recipient's flank.

The above-described methods were used to obtain the following results:

5        I.        SUPPRESSION OF THE MIXED LYMPHOCYTE REACTION (MLR) BY ORAL ADMINISTRATION OF ALLOGENEIC SPLENOCYTE PREPARATIONS

Splenocytes from WF rats were freshly prepared and were administered orally to LEW rats two, five or ten times over a 1-2 week period.

10        The individual dosages were 50 million cells per oral administration.

15        Seven days following the last oral administration, lymph nodes were taken from both a control group and those given oral splenocytes for MLR studies using WF or BN stimulators. As shown in Figure 1, LEW rats which had ingested allogeneic splenocytes showed significantly reduced reaction against the lymphocytes from the WF strain. This phenomenon was observed in all three feeding protocols (i.e., 2, 5 or 10 times). However, only the group that received ten feedings 20        showed suppression against the BN strain, the third party control.

These results indicate that limited ingestion of allogeneic splenocyte preparations induced antigen specific suppression of the MLR.

25

II.        COMPARISON OF SUPPRESSION OF MLR BY ORAL ADMINISTRATION OF ALLOGENEIC VERSUS SYNGENEIC SPLENOCYTE PREPARATIONS

30        A dose response study was subsequently conducted to determine the effect of feeding syngeneic versus allogeneic

cells. LEW rats were fed twice with 1, 5, 25 or 50 million splenocytes from either LEW or WF strains. The results are set forth in Table I below.

TABLE I

5

The Effect of Feeding Syngeneic  
and Allogeneic Splenocytes on MLR

	Strains used for feeding	Dosage cells/feeding	MLR delta/CPM	Relative Response (%)
10	----	----	115015±7707	100
LEW	1 x 10 <sup>6</sup>		128520±8338	112
	5 x 10 <sup>6</sup>		54391±10988	47
	25 x 10 <sup>6</sup>		39088±7294	34
	50 x 10 <sup>6</sup>		81329±8013	71
WF	1 x 10 <sup>6</sup>		71135±13721	62
	5 x 10 <sup>6</sup>		79011±14119	68
	25 x 10 <sup>6</sup>		56196±15254	49
	50 x 10 <sup>6</sup>		73541±11636	64

20

Feeding at the lowest dosage (1 million) of syngeneic cells did not induce suppression; all other doses, both syngeneic and allogeneic cells, show some suppression to varying degrees.

25

III. EFFECT OF INGESTED LYSATE OF ALLOGENEIC SPLENOCYTE PREPARATIONS ON MLR

The effect of ingested lysate alone on MLR was next studied to determine whether live splenocytes were required for

the orally induced tolerance. Rats were given two separate oral doses of either live splenocytes or the corresponding lysate prepared by the repetitive freeze and thaw method (described above) and the effect of these treatments were compared. Figure 2 shows that cell lysate alone was sufficient in suppressing the MLR, indicating that a subcellular fragment was involved in suppressing the cell-mediated immunity.

IV. KINETICS OF MLR SUPPRESSION BY ORAL ADMINISTRATION OF ALLOANTIGENS

The kinetics of the orally induced tolerance to alloantigen was studied by giving two oral doses of splenocytes to separate LEW rat groups, 14 days, 7 days, 3 days, and 1 day before the MLR was performed. As shown in Figure 3, the groups which were given oral doses 1 day or 3 days before MLR was performed did not induce suppression. The groups with 7-day and 14-day intervals between the last oral ingestion and MLR showed dramatic reduction of proliferation in MLR, indicating that more than 4 days were required for the induction of oral unresponsiveness to alloantigens.

V. SUPPRESSION OF DTH RESPONSE AGAINST ALLOANTIGENS

In addition to the in vitro MLR, the effect of ingesting allogeneic splenocytes on the delayed type hypersensitivity (DTH) response, in vivo, in LEW rats was examined. LEW rats were orally administered 10 feedings of 50 million splenocytes from either syngeneic or allogeneic (WF) animals. After the last oral ingestion, the test for DTH was initiated with the animals being immunized subcutaneously in their foot pads. The

same animals were injected again 10 days later in the ear lobes. The DTH was measured as the changes in the ear thickness before and 48 hours after the challenge. The results are shown in Figure 4.

5       Approximately 50% decrease in DTH response to WF was observed in rats fed with cells of the same strain, but not in those fed with syngeneic LEW splenocytes. The DTH response against BN was not affected by the pre-treatment, indicating that the DTH suppression was antigen specific.

10      VI.     ACTIVE SUPPRESSION IS INVOLVED IN MEDIATING DECREASED PROLIFERATION IN THE MLR

15      In order to study the mechanism of inhibition of MLR proliferation in the fed animals, a suppressor cell assay was performed to determine if CD8+ suppressor cells were involved in mediating the observed effects. Lymphocytes from either control or pre-fed animals were irradiated with 1000 RADS of gamma radiation before being added to a primary MLR, serving as modulators.

20      Lewis rats (3/group) were pre-treated 10 times orally with varying dosages (as indicated in the Table) of WF splenocytes. One week later, their cervical lymph nodes were taken and the cells served as modulator after being irradiated 1000 Rad of gamma radiation. The primary LEW anti-WF and LEW anti-BN MLR and Con-A stimulation cultures were set up as described above. Modulator cells were added to the primary cultures at a 1/5 ratio. The results are set forth in Table II below.

TABLE II

SUPPRESSION OF PRIMARY MLR BY LYMPHOCYTES FROM  
ALLOGENEIC SPLENOCYTES FED RATS

5	Source of modulator	anti-WF		anti-BN		Con A	
10		CPM ( $\times 10^{-3}$ )	% Supp.	CPM ( $\times 10^{-3}$ )	% Supp.	CPM ( $\times 10^{-3}$ )	% Supp.
15	--	112 $\pm$ 21		405 $\pm$ 78		280 $\pm$ 4.7	
	control	104 $\pm$ 11	7	464 $\pm$ 15	0	276 $\pm$ 6.6	1.4
	fed 10 $\times$ 10 <sup>6</sup>	106 $\pm$ 17	5.3	334 $\pm$ 120	17	277 $\pm$ 34	1
	fed 25 $\times$ 10 <sup>6</sup>	84 $\pm$ 15	25	443 $\pm$ 17	0	305 $\pm$ 3.9	0
	fed 50 $\times$ 10 <sup>6</sup>	1 $\pm$ 0.9	99	84 $\pm$ 7.1	79	199 $\pm$ 12	29

20 The results in Table II show that adding 20% of modulators from pre-fed animals, but not from the control animals, suppressed the primary LEW-anti-WF MLR. This suggests that suppressor cells were induced after feeding and these in turn mediated suppression of the MLR.

VII. PHENOTYPE OF LYMPH NODE CELLS FROM  
FROM ANIMALS INGESTING SPLENOCYTES

25 Cervical lymphocytes from either control or fed animals were cultured with irradiated WF stimulators for 5 days, then sorted for CD4+ or CD8+ cells by indirect immunofluorescence staining. The results shown in Figure 5 show that pre-feeding rats with allogeneic splenocytes resulted in an increase in CD8+ (suppressor T-cells) cells and a decrease in CD4+ (helper T-cells) cells when compared to controls.

VIII. ORAL ADMINISTRATION OF SPLENOCYTES PREVENTS ACCELERATED CARDIAC ALLOGRAFT REJECTION

To demonstrate the prevention of allograft rejection, 5 an accelerated rejection transplantation model, as described above, was used. LEW rats were pre-sensitized with BN skin grafts 7 days before challenge with vascularized BN test cardiac allografts, to study the effects of feeding allogeneic donor splenocytes on test graft survival.

10 While unsensitized controls rejected their cardiac allografts on the 6th through the 8th day, all sensitized control animals hyperacutely rejected their cardiac allografts within 36 hours. Test animals fed 5-10 feedings of 50 million splenocytes, 7 days prior to the skin graft, or even on the day 15 of the skin graft, exhibited increased test cardiac allograft survival, to  $7.62 \pm 0.5$  days.

These results show that feeding allogeneic splenocytes prevents sensitization and converts accelerated rejection into an acute form.

20 The specificity of this phenomenon was examined as described below.

Cardiac recipient LEW rats were either unfed (n=10), fed LEW (syngeneic) lymphocytes (n=8), fed BN splenocytes (but received a WF cardiac allograft, n=6) or were fed BN 25 splenocytes (and received a BN cardiac allograft, n=8). All fed animals received 5-10 feedings of  $50 \times 10^6$  splenocytes.

The results are shown in Figure 6.

As can be seen in Figure 6, only the rats which were fed allogeneic splenocytes showed cardiac allograft survival beyond day 3. LEW rats fed third party (BN) lymphocytes but receiving a WF graft did not demonstrate enhanced cardiac allograft survival, demonstrating the specificity of this reaction.

In a preliminary attempt to study the mechanism of graft prolongation, the MLR of cervical lymph node cells from control and fed sensitized LEW rats were examined at 48 hours after the cardiac transplant. The results are shown in Figure 7.

There was a suppression of the MLR in the fed animals as compared to the control (Figure 7). These data are consistent with the previous MLR findings in the naive animal model.

WHAT IS CLAIMED IS:

1        1. A method for suppressing the immune response of a  
2        recipient mammal to the presence of non-self tissue from a  
3        donor mammal comprising:

4                orally or enterally administering to a mammal in  
5        need of such treatment an immune suppressive effective amount  
6        of an agent specific for suppressing said immune response.

1        2. The method of claim 1 wherein said agent is a  
2        member selected from the group consisting of splenocytes from  
3        said donor, splenic extracts from said donor, cultured lym-  
4        phocytes from said donor, extracts of said cultured lym-  
5        phocytes, MHC antigens from said donor, disease suppressive  
6        fragments of said MHC antigens and analogs thereof from said  
7        donor.

1        3. A pharmaceutical formulation for administration to  
2        a recipient mammal about to undergo or having undergone  
3        transplant surgery from a donor mammal, comprising:

4                an oral dosage form containing an effective amount  
5        for suppressing transplantation rejection of an agent selected  
6        from the group consisting of splenic tissue from said donor,  
7        extracts of said splenic tissue, cultured lymphocytes from said  
8        donor, extracts of said cultured lymphocytes, transplantation  
9        MHC antigens from said donor, disease suppressive fragments of  
10      said MHC antigens and analogs thereof.

1        4. The pharmaceutical formulation of claim 3 wherein  
2        said oral dosage form is a solid dosage form selected from the  
3        group consisting of a tablet, a capsule and a caplet.

1        5. The pharmaceutical formulation of claim 3 wherein  
2        the oral dosage form comprises an aqueous suspension solution.

1        6. The pharmaceutical formulation of claim 3 comprising  
2        a pharmaceutically acceptable carrier or diluent.

1        7. A method for suppressing the immune response of a  
2        recipient mammal to the introduction of a donor's foreign  
3        tissue comprising:

4                aerosol administering to a mammal in need of such  
5        treatment an immune suppressive effective amount of an agent  
6        selected from the group consisting of splenocytes from said  
7        donor, splenic extracts from said donor, cultured lymphocytes  
8        from said donor, extracts of said cultured lymphocytes, MHC  
9        antigens from said donor, disease suppressive fragments of MHC  
10        antigens and analogs thereof.

1        8. A pharmaceutical formulation for administration to  
2        a recipient mammal about to undergo or having undergone  
3        transplant surgery from a donor mammal, comprising:

4                an aerosol dosage form containing an effective  
5        amount for suppressing transplantation rejection of an agent  
6        selected from the group consisting of splenic tissue from said  
7        donor, extracts of said splenic tissue, cultured lymphocytes

8       from said donor, extracts of said cultured lymphocytes,  
9       transplantation MHC antigens from said donor, disease suppres-  
10      sive fragments of said MHC antigens and analogs thereof.

1           9. The pharmaceutical formulation of claim 8 wherein  
2       the aerosol dosage form comprises an aqueous suspension  
3       solution.

1           10. The pharmaceutical formulation of claim 8 compris-  
2       ing a pharmaceutically acceptable carrier or diluent.

1           11. A method for suppressing graft rejection in a  
2       mammal comprising administering to a mammal in need of such  
3       treatment an effective amount of an agent which specifically  
4       suppresses said mammals immune response directed against said  
5       graft.

1           12. An oral dosage form for administration to a mammal  
2       comprising a composition containing an agent that specifically  
3       suppresses said mammal's immune response directed against a  
4       transplanted organ or tissue.

1           13. A dosage form for administration to a mammal via  
2       the oral cavity comprising a composition containing an agent  
3       which specifically suppresses said mammal's immune response  
4       directed against a transplanted organ or tissue.

1/3-

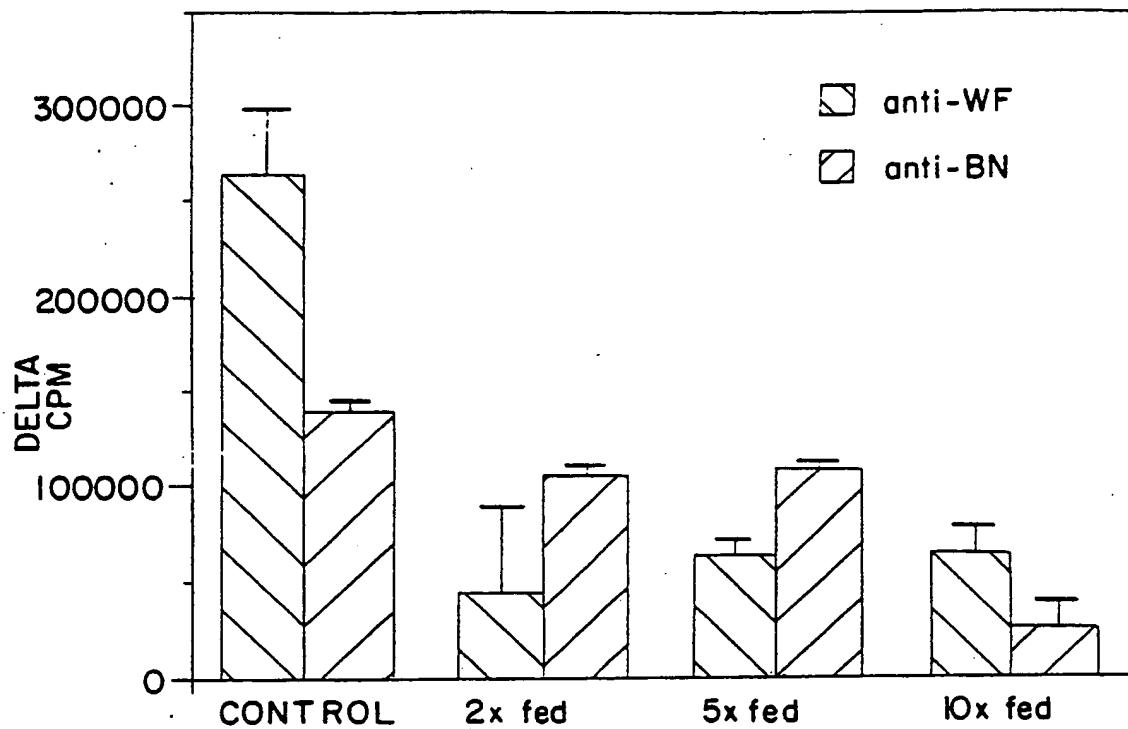


FIG. 1

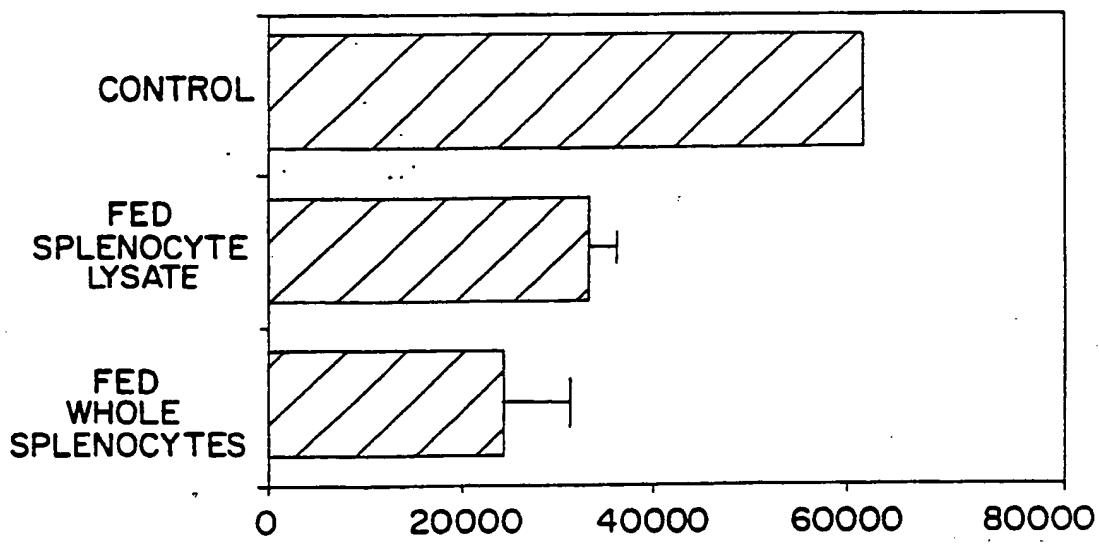


FIG. 2

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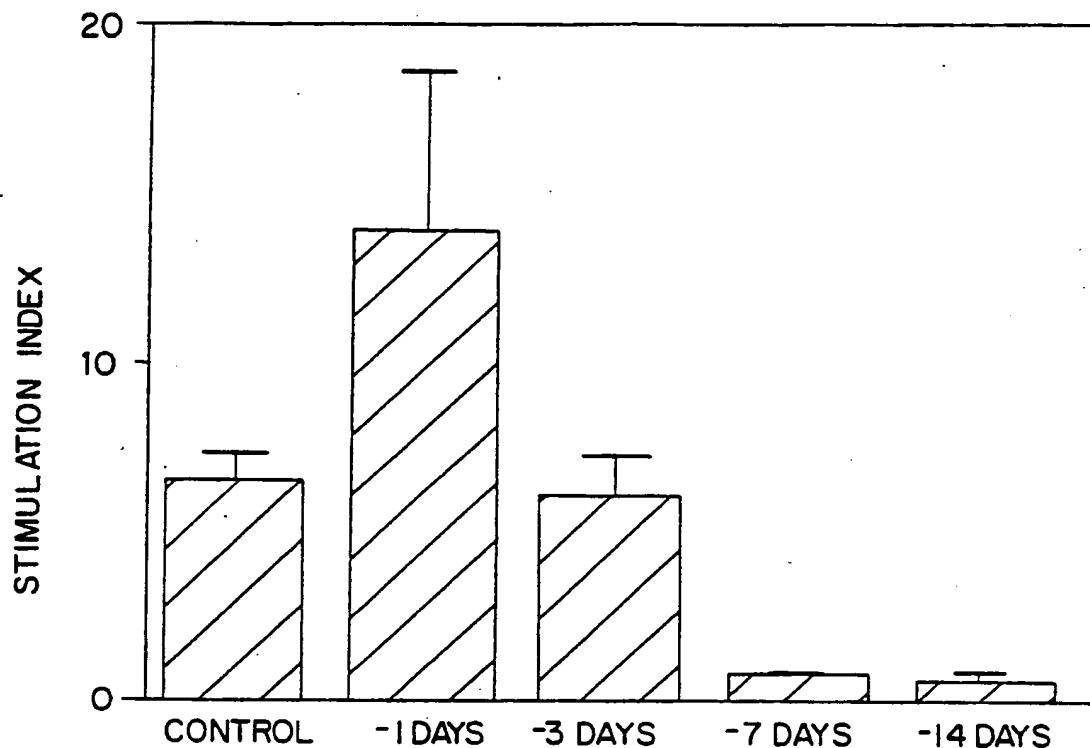


FIG. 3

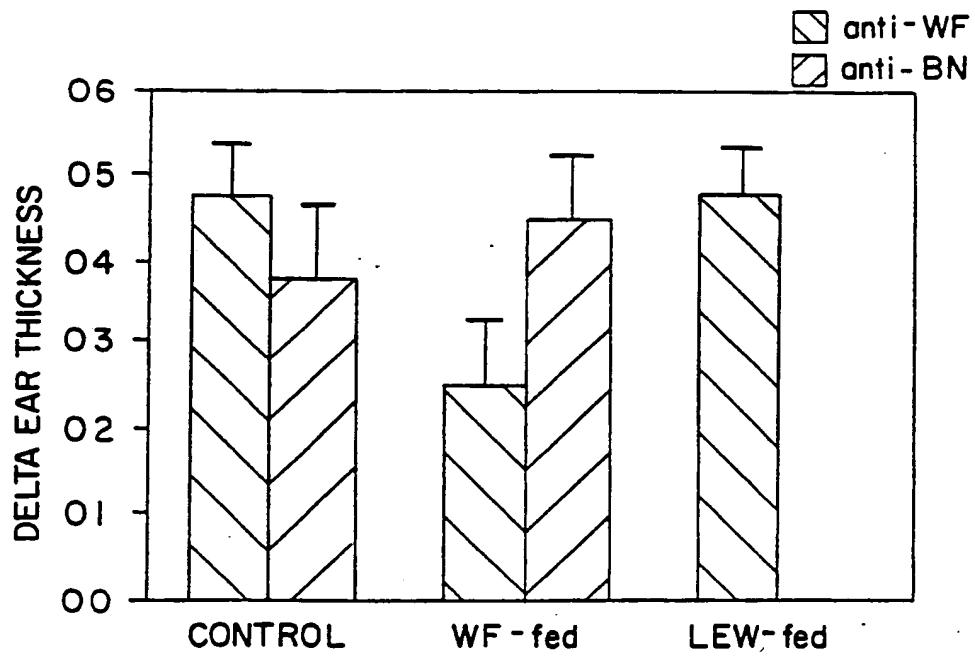
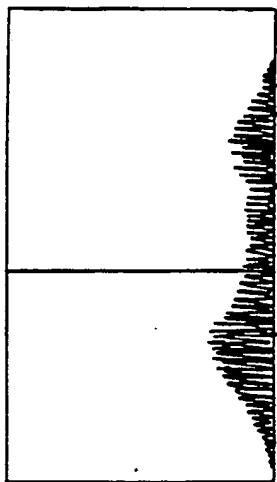


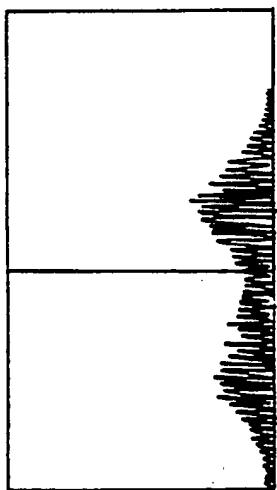
FIG. 4

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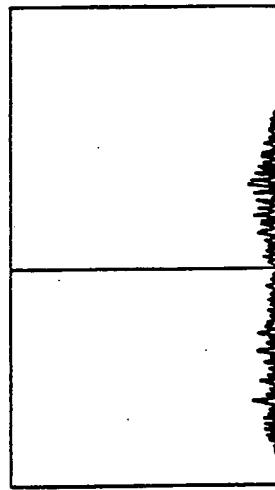
3/5



CD8<sup>+</sup>



CONTROL



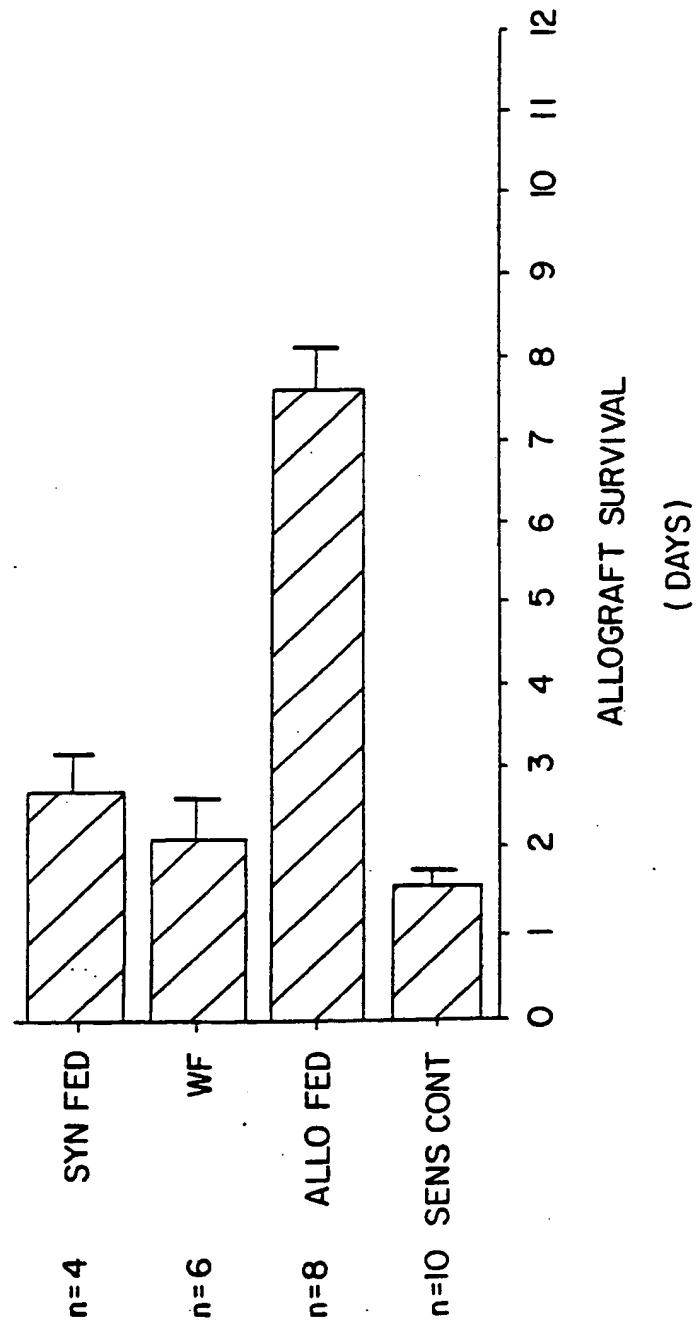
FED

CD4<sup>+</sup>

FIG. 5

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FIG. 6



**SUBSTITUTE SHEET**

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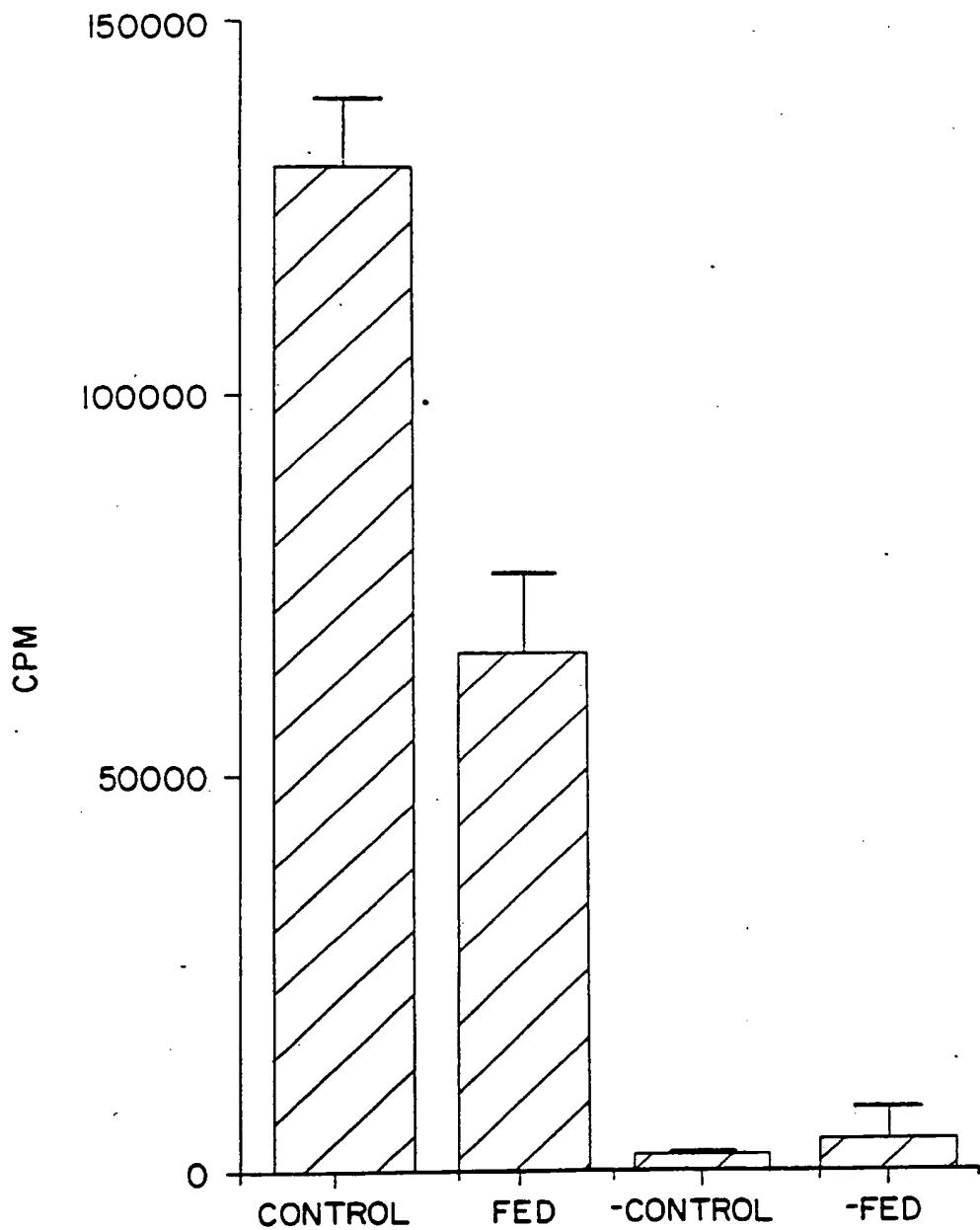


FIG. 7

# INTERNATIONAL SEARCH REP RT

International Application No. PCT/US91/08143

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC  
 IPC(5) ~~2~~ A61F 39/00, 37/10, 37/00

U.S.CL: 424/88; 514/8, 21

## II. FIELDS SEARCHED

Minimum Documentation Searched ?

Classification System	Classification Symbols
U.S.	424/88; 514/8, 21

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

Databases: Dialog (Files 5, 155, 154, 399, 357, 172, 173), USPTO  
Automated Patent System (File USPAT, 1971-1991).

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages ***	Relevant to Claim No. 13
Y	Transplantation, Volume 39, No. 1, issued January 1985, Wood et al., "Suppression of Renal Allograft Rejection In the Rat By Class I Antigens On Purified Erythrocytes," pages 56-62, see abstract.	1-13
Y	The Journal of Immunology, Volume 121, No. 6, issued December 1978, Richman, et al., "Enterically Induced Immunologic Tolerance I. Induction of Suppressor T Lymphocytes by Intragastric Administration of Soluble Proteins," pages 2429-2434, see abstract.	1-13

- \* Special categories of cited documents: \*\*
- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "D" document referring to an oral disclosure, use, exhibition or other means
- "E" document published prior to the international filing date but later than the priority date claimed

- "F" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "G" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "H" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "I" document member of the same patent family

## IV. CERTIFICATIONS

Date of the Actual Completion of the International Search

02 January 1992

Date of Mailing of this International Search Report

10 Feb 1992

International Searching Authority

ISA/US

Signature of Authorized Officer

Lynette F. Smith

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	The Journal of Immunology, Volume 140, No. 2, issued 15 January 1988, Higgins et al., "Suppression of Experimental Autoimmune Encephalomyelitis by Oral Administration of Myelin Basic Protein and Tts Fragments", pages 440-445, see entire article.	1-13
Y	Clarke and Davis, "Aerosols and The Lung" published 1984, see pages 197-224, especially pages 199 and 218.	1-13